a1 Cont

Serial No. 08/259,609, filed June 14, 1994, now U.S. Patent No. 5,989,808, which are incorporated by reference herein.

Replace the second paragraph on page 9 with the following paragraph:

a2

Figures 2A and 2B are photographs of plates which show the results of the growth experiments conducted in Example 1 for stains CY722, CY723, CY724, and CY781 on non-selective medium and selective medium, photographs A and B, respectively.

Replace the paragraph bridging pages 25 and 26 with the following paragraph:

a3

Oligonucleotides prepared on an ABI oligosynthesizer are designed according to the published cDNA sequence for pig GH (*see* Su and El-Gewely, 1988). A 30 base 5' oligonucleotide contains a NcoI site (5'-CATGCCATGGAGGCCTTCCCAGCCATGCCC 3') (SEQ ID NO: 1) and a 27 base 3' oligonucleotide contains a BamHI site (5'-CGGGATCCGCAACTAGAAGGCACAGCT-3') (SEQ ID NO: 2). The GH cDNA is generated using a pig pituitary lambda gt11 library as template source. A 540 bp fragment is obtained, ligated into pCR II vector (Invitrogen Corp.), recombinants are confirmed by restriction enzyme digest, and the DNA produced as described in Maniatus *et al.*, 1982. The cDNA sequence is confirmed by di-deoxy terminator reaction using reagents and protocols from Perkin-Elmer Cetus Corp. and an ABI 373A automated sequencer. The GH cDNA is directionally cloned into pACT-II via NcoI and BamHI sites. The cDNA encoding the extracellular domain of the GHR is generated using standard PCR methods. A 33 base 5' oligonucleotide containing a NcoI site (5'-CATGCCATGGAGATGTTTCCTGGAAGTGGGGCT-3') (SEQ ID NO: 3) and a 39 base 3' oligonucleotide containing a termination codon, followed by a NcoI site

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(5'-CATGCCATGGCCTACCGGAAATCTTCTTCACATGCTGCC-3') (SEQ ID NO: 4) are used to generate a 742 bp fragment encoding amino acids 1-247 of the rat GHR (Baumbach *et al.*, 1989). This GHR cDNA is cloned into vector pCRII as previously described above, and then subcloned into the NcoI site of vector pAS2. DNA of the final recombinant vectors is transformed into yeast strain(s) by the lithium acetate method (Rose *et al.*, 1990).

Replace the paragraph bridging pages 27 and 28 with the following paragraph:

To substantiate the apparent binding of GH to its receptor in the foreign environment of a yeast nucleus, the system is modified to add a third plasmid mediating expression of "free" ligand to show that the GH peptide competes with the GH-Gal4 fusion protein, reversing the 2-hybrid interaction shown in Example 1. The parental strain Y190 (Wade Harper *et al.*, 1993) is grown on a medium containing 5-fluoro-orotic acid to select for derivatives that spontaneously lose the URA3 gene (see Rose *et al.*, 1990). The resultant strain, designated CY770, is utilized for all experiments examining the effects of protein expressed concurrently from the third component, (that is, the third plasmid). The cDNA encoding GH is generated by PCR methods using
a 38 base 5' oligonucleotide containing an EcoRI site
(5'-CCGAATTCAAAATGGCCTTCCCAGCCATGCCCTTGTCC-3') (SEQ ID NO: 5) and
a 26 base 3' oligonucleotide containing a HindIII site
(5'CCAAGCTTCAACTAGAAGGCACAGCT-3') (SEQ ID NO: 6) for subsequent subcloning into the vector pCUP. pCUP is an inducible yeast expression vector derived from pRS316 (Hill *et al.*, 1986). Briefly, this vector is constructed by inserting the 3' end of the yeast PGK gene (from pPGK; Kang *et al.*, 1990) into the pRS316 cloning region

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as a BamHI-SalI fragment to serve as a transcriptional terminator. To this plasmid, the CUP1 promoter region (Butt *et al.*, 1984) is amplified by PCR as a SacI-EcoRI fragment and inserted into corresponding sites of the plasmid to create pCUP. The GH expression plasmid (GH-pCUP) is then co-transformed with the GH and GHR fusion constructs into strain CY770 to generate CY781. Concurrent expression of free GH with the GH and GHR fusion proteins (CY781) is shown to block GH-GHR-dependent cell growth on selective medium (Figure 2B). This experiment typifies an *in vivo* competition assay and demonstrates the reversibility of the observed ligand-receptor interaction.

Replace the first paragraph on page 29 with the following paragraph:

To expand and validate this technology, a similar system was developed using the peptide hormone prolactin (PRL) and its receptor. Prolactin is structurally related to GH and the prolactin receptor (PRLR) is also a member of the cytokine receptor superfamily. Unlike human GH, sub-primate GH does not readily bind the PRLR (Young and Bazer, 1989); nor does PRL readily bind the GHR (Leung *et al.*, 1987). Mature porcine PRL is generated as a fusion to the GAL4 activation domain. Oligonucleotides are designed to pig PRL (obtained from GenBank; Accessign No. X14068), and used to generate the mature pig PRL protein hormone from a pig pituitary lambda gt11 library, using standard PCR methods. A 31 base 5' oligonucleotide includes an EcoRI site (5'-CGGAATTCTGCCCATCTGCCCCAGCGGGCCT-3') (SEQ ID NO: 7) and corresponds to sequences encoding amino acids 1-7. A 30 base 3' oligonucleotide contains an EcoRI site

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(5'-GAATTCACGTGGCTTAGCAGTTGCTGTCG-3') (SEQ ID NO: 8) and corresponds to a region of cDNA 3' to the endogenous termination codon. A 600 bp fragment is obtained, ligated into vector pCR II, and confirmed by restriction enzyme digest and sequence analysis. The PRL cDNA is cloned into pACT-II via the EcoRI site.

a5 cont

Replace the paragraph bridging pages 29 and 30 with the following paragraph:

The extracellular domain of the porcine PRL receptor (PRLR) is generated as a fusion to GAL4 DNA binding domain. Oligonucleotides are designed based on sequence of the mouse PRLR (Davis and Linzer, 1989) A 31 base 5' oligonucleotide contains a SmaI site (5'-TCCCCCGGGGATGTCATCTGCACTTGCTTAC-3') (SEQ ID NO: 9) while the 31 base 3' oligonucleotide contains a termination codon followed by a SalI site (5'TCCGTCGACGGTCTTTCAAGGTGAAGTCATT-3') (SEQ ID NO: 10). These oligonucleotides flank the extracellular domain of the PRLR, encoding amino acids 1-229. A pig pituitary lambda gt11 library is used as a template source. Using standard PCR methods, a 687 bp fragment is generated, ligated into pCRII, and the nucleotide sequence is confirmed. The PRLR cDNA is cloned into the pAS2 vector via the SmaI and SalI restriction sites.

a6

Replace the first paragraph on page 31 with the following paragraph:

Additional strains are developed to assess ligand-receptor specificity. URA- strains expressing GH and GHR fusion proteins are transformed with pCUP or PRL-pCUP, while strains expressing PRL and PRLR fusion proteins are transformed with pCUP, or PRL-pCUP. Briefly, PRL-pCUP is constructed in a fashion similar to that described for GH-pCUP. The PRL cDNA is generated by PCR using a 33 base 5' oligonucleotide with an EcoRI site

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(5'-GAATTCAAAATGCTGCCCATCTGCCCCAGCGGG-3') (SEQ ID NO: 11) and the 3' oligonucleotide in example 1B. The resulting fragment is introduced into pCUP via the EcoRI site. As demonstrated in the above Examples, a strain expressing the GH and GHR fusions with no competitor grows on selective medium and this growth is abolished with coexpression of free GH. The prolactin experiment produces similar results, which confirms the specificity of the ligand-receptor binding in the yeast cell. A strain carrying PRL and PRLR fusions (CY787) can grow on selective medium and this growth is abrogated by expression of free PRL (CY786; Table 1).

a7 cont

Replace the paragraph bridging pages 33 and 34 with the following paragraph:

Low-copy-number plasmids expressing GHR- or GH-Gal4 fusion proteins (pOZ153 and pOZ152, respectively) are constructed to reduce expression of these proteins. In addition, a novel reporter gene is constructed that prevents cell proliferation on selective medium unless expression is abrogated. To construct the GHR fusion expression plasmid, a SacI-BamHI restriction fragment containing a yeast constitutive promoter and GAL4 sequences is isolated from pAS1 (Durfee *et al.*, 1993) and cloned into pUN30 (Elledge and Davis, 1988). The extracellular domain of GHR is then fused to GAL4 by ligation as an NcoI fragment as described in Example 1 to create pOZ153. To construct the GH fusion expression construct the entire GH-Gal4 region with promoter and terminator sequences is isolated from the plasmid described in Example 1 as a PvuI-SalI fragment. This DNA segment is cloned into pUN100 (Elledge and Davis, 1988) generating pOZ152. A reporter gene is constructed by isolating the yeast CYH2 coding region and operatively linking it to a GAL promoter in a yeast expression plasmid. Briefly, the GAL1 promoter region is inserted into YEp352 (Hill *et al.*, 1986) as

a8

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a 685 bp EcoRI-BamHI fragment. CYH2 sequences are amplified by PCR using oligonucleotides primers (5'-GGATCCAATCAAGAATGCCTTCCAGAT-3' (SEQ ID NO: 12) and 5'-GCATGCGTCATAGAAATAATACAG-3' (SEQ ID NO: 13)) and pAS2 as the template. The PCR product is digested with BamHI plus SphI and cloned into the corresponding sites in the YEp352-GAL vector. These plasmids are transformed into yeast strain CY770 which carries a mutation at the chromosomal cyh2 gene rendering the strain resistant to the protein synthesis inhibitor cycloheximide. The presence of all three plasmids is necessary to confer cycloheximide sensitivity (cyh^s).

a8 cont

Replace the paragraph bridging pages 36 and 37 with the following paragraph:

The interaction of vascular endothelial cell growth factor (VEGF) with the ligand binding domain of its cognate receptor (KDR, kinase insert domain containing receptor) is described as an example for this system. KDR is a tyrosine kinase receptor, and dimer formation (1 ligand - 2 receptors) is suggested to be important for hormone-induced receptor function. The cDNA encoding the ligand domain of KDR (Terman *et al.*, 1991) is isolated as an NCO I - BamHI fragment and cloned into both the pACT-II and pAS2 vectors. The cDNA encoding the mature protein for VEGF is generated using standard PCR techniques. Oligonucleotides are designed from published sequence (see Fischer *et al.*, 1991). A 34 base 5' oligonucleotide containing an EcoRI site (5'-CGGAATTCGAAGTATGGCACCCATGGCAGAAGGA-3') (SEQ ID NO: 14) and a 28 base 3' oligonucleotide containing an EcoRI site (5'-CGGAATTCGGATCCTCATTCATTCATCA-3') (SEQ ID NO: 15) are used to generate a 450 bp fragment encoding the mature protein and cloned into the EcoRI site

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a9 Cont

of pCUP. DNA of final recombinant vectors is transformed into yeast by the lithium acetate method to generate appropriate strains.

Replace the paragraph bridging pages 39 and 40 with the following paragraph:

a10

The luciferase reporter plasmid(s) for use in yeast two-hybrid applications were generated as follows. Plasmid pEK1 (described in Price et al., 1995) was digested with BamHI+SalI restriction enzymes, dephosphorylated, and gel purified. The luciferase coding region was obtained as a Bgl II – Sal I 1.9 kb cDNA fragment from pGL3Basic (Promega), purified, and ligated to the prepared pEK1 vector using standard procedures to generated plasmid Kp126. The recombinant DNA is transformed into DH5α *E. coli* using standard procedures and plasmid DNA prepared. The construct was confirmed by restriction enzyme analysis and cDNA sequence analysis (using primer Kx38: 5'-TCAAATTAACAACCATAGGAT-3') (SEQ ID NO: 16). Kp126 was used extensively for yeast two-hybrid systems and retains the original mammalian Kozak sequence from original DNA source.

Replace the first full paragraph on page 43 with the following paragraph:

a11

The complete ORFs for G-alphaZ and RGS-Z were isolated from a human brain cDNA library (Quickclone cDNA, Clontech) by PCR amplification. PCR primers were designed 5' and 3' of the open reading frame of G-alphaZ (GenBank#J03260) and RGS-Z (Genbank#AF074979). PCR amplification was performed under standard buffer conditions using the Clontech cDNA Advantage cDNA kit. The primers were G-alphaZ-fwd 5' ACCATGGGATGTCGGCAAAGCTCAGAGGAAA-3' (SEQ ID NO: 17) and G-alphaZ-rev 5'-CAAGGGGTGGGGGACATT-3' (SEQ ID NO: 18) for G-alphaZ and RGS-Z-fwd 5'-CCCGGCCGGCAGGTGGAC-3' (SEQ ID NO: 19) and RGS-Z-rev 5'-

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CTCATGCAAAATAAAAGTGGTTC-3' (SEQ ID NO: 20) for RGS-Z. Cycle parameters were 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute for a total of 30 cycles.

a11 Cont

Replace the second full paragraph on page 44 with the following paragraph:

A constitutively active (Q205L; Wang et al., 1998) form of G-alphaZ was generated. The Q205L mutation in G-alphaZ was generated in the GaZ-pGBKT7 and GaZ-pACT2 plasmids using the QuickChange kit (Stratagene) and the primers 5'-GTGGGGGGCTGAGGTCAGAG-3' (SEQ ID NO: 21) and 5'-CTCTGACCTCAGCCCCCCAC-3' (SEQ ID NO: 22). Recombinant mutant plasmids were transformed into bacterial cells and DNA isolated using standard methods. The Q205L G-alphaZ mutants were identified by sequence analysis of the resulting colonies and termed Q205L/GaZ-pGBKT7 and Q205L/GaZ-pACT2.

a12

On page 54, replace the heading for Example 10 with the following:

a13

Example 10: Multiplex assays using strains expressing different luciferase reporter genes (Firefly Luc reporters and Renilla Luc reporter)

<u>In the Claims</u>:

Please amend the claims as follows:

7. (Amended) The yeast cell of claim 6 wherein the peptide is a growth factor selected from the group consisting of epidermal growth factor, nerve growth factor, leukemia inhibitory factor, fibroblast growth factor, platelet-derived growth factor, vascular endothelial growth factor, tumor necrosis factor, oncostatin M, ciliary neurotrophic factor, erythropoietin, steel factor, placental lactogen, and transforming growth factor β ("TGF").

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